

Protein accumulation and composition in wheat grains: Effects of mineral nutrients and high temperature

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Abstract

Effects of mineral nutrition and temperature on accumulation and composition of protein in wheat grains and on baking quality were studied under controlled environments. Under a moderate temperature regimen of 24 °C days and 17 °C nights (24/17 °C), post-anthesis N:P:K 20:20:20 (NPK) supplied by continuous drip irrigation increased the rate of protein accumulation, doubled flour protein percentage and slightly increased final single kernel weight. In contrast, post-anthesis NPK had almost no effect on rate or duration of protein accumulation or flour protein percentage under a high temperature regimen of 37 °C days and 28 °C nights (37/28 °C). The 37/28 °C regimen shortened and compressed the stages of grain fill, reduced the duration of dry matter accumulation, and reduced single kernel weight by 50%. Rate and duration of protein accumulation in thermal time and total protein per grain at 37/28 °C with or without NPK were similar to that at 24/17 °C in the absence of post-anthesis NPK. Protein percentage was higher for flour from grain produced at 37/28 °C with or without NPK than at 24/17 °C in the presence of post-anthesis NPK. Transcript and protein profiling studies confirmed that the 37/28 °C regimen compressed development without disrupting coordinate synthesis of gliadins and glutenin subunits, although some specific effects of NPK and temperature on relative amounts of individual gliadins and glutenins were observed. Transcript levels for ω -gliadins, α -gliadins and high molecular weight glutenin subunits (HMW-GS) declined at 24/17 °C in the absence of post-anthesis NPK, whereas transcript levels for low molecular weight glutenin subunits (LMW-GS) and γ -gliadins showed little change. Two-dimensional gel electrophoresis (2DE) demonstrated that relative spot volumes for several ω -gliadins, α -gliadins and HMW-GS were lower at 24/17 °C in the absence than in the presence of post-anthesis NPK, whereas the relative spot volume for a major LMW-GS was lower in the presence of NPK. Effects of temperature on relative spot volume were generally smaller than effects of NPK. Compared to the 24/17 °C regimen in the absence of post-anthesis NPK, relative spot volume for some α -gliadins and HMW-GS were higher at 37/28 °C, with or without NPK, and relative spot volume for a major LMW-GS decreased at 37/28 °C. Loaf volume was correlated with flour protein percentage regardless of temperature regimen but mixing tolerance was highest for flour from grain produced under the 24/17 °C regimens with NPK.

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1. Introduction

Improving N use efficiency is of great economic and environmental importance, particularly for wheat (Raun and Johnson, 1999). Flour protein content is one of the primary determinants of bread making quality and is directly related to grain protein concentration, which is determined by complex interactions between genetics, environment and N availability (Asseng et al., 2000; Fowler et al., 1990; Panozzo and Eagles, 1999).

Frequently there is a negative relationship between grain yield and protein content (Fowler, 2003; Pleijel et al., 1999). Prior to anthesis, yield and grain protein content are influenced by effects of genetics, environment, N fertilization and other aspects of crop management on such factors as stand density, root growth, number of tillers, and number of florets per head (Bahrman et al., 2004). After anthesis, kernel growth is directly impacted by soil and air temperature, water and N, as well as source–sink relations with leaves and stems. Adding post-anthesis N may directly increase grain protein content without reducing yield, whereas post-anthesis heat or drought may increase grain protein content but reduce yield because of their effects on starch production (Bhullar and Jenner, 1985; Cassman et al., 1992;

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Fowler, 2003). Understanding the interactions between N, heat and drought during grain fill are important for deciding how and when to add additional N fertilizer.

Protein composition is a major determinant of breadmaking quality that also may be influenced by N and temperature. The gluten storage proteins, composed of the monomeric gliadins and the high and low molecular weight glutenin subunits (HMW-GS and LMW-GS) of the glutenin polymer, are essential to breadmaking properties (Payne, 1987). There are some indications that increased grain protein content achieved by N fertilization or high growing temperatures is accompanied by changes in proportions of gliadins and glutenins, but the reports are not consistent and leave many unanswered questions. Several reports indicate that proportions of gliadins increased with increasing flour protein and proportions of glutenin decreased (Doekes and Wennekes, 1982; Gupta et al., 1992; Triboui et al., 2000; Wieser and Seilmeier, 1998). Albumins and globulins did not increase proportionately to increased flour protein in response to added N or increased temperature (Doekes and Wennekes, 1982; Gupta et al., 1992; Pence et al., 1954; Triboui et al., 2003; Wieser and Seilmeier, 1998). In general, increased flour protein correlates with improved breadmaking quality, including increased loaf volume (Finney and Fryer, 1958; Graybosch et al., 1996; Johansson et al., 2001; Randall and Moss, 1990). However, changes in protein composition associated with increased exposure to temperatures above 30 °C were reported to be related to more rapid dough breakdown (Corbellini et al., 1998; Gibson et al., 1998; Panozzo and Eagles, 2000), decreased loaf volume (Finney and Fryer, 1958) or decreased SDS sedimentation scores (Graybosch et al., 1995).

Several studies used improved separation techniques to examine the effects of N or temperature on specific gliadin and glutenin types. Wieser and Seilmeier (1998) reported that proportions of ω -gliadins and HMW-GS glutenins in flour protein increased and proportions of LMW-GS and γ -gliadins decreased with N fertilization. Daniel and Triboui (2000) reported that proportions of ω -gliadins increased with added N and increased temperature. Proportions of α -gliadins decreased with N and increased with temperature, while γ -gliadins increased with N and decreased with temperature. Luo et al. (2000) reported no change in ratio of HMW-GS to LMW-GS with added N and S, and Triboui et al. (2000) reported no significant change in ratio of HMW-GS to LMW-GS in response to added N. Except for individual HMW-GS, no report quantified changes in the 100 or more individual gliadins and glutenin subunits present in any one wheat variety. The effects of environment on profiles of protein accumulation in developing wheat grains also were examined by 2DE (Majoul et al., 2003, 2004) but effects on individual gliadin and glutenins resolved by 2DE have not been reported.

Field plots give realistic phenotypes under unpredictable conditions, whereas controlled environments give consistent phenotypes under predictable conditions, both of which are useful for testing models of grain fill. Controlled environments are also essential for establishing reproducible conditions for studying the underlying molecular mechanisms by which environment influences wheat grain protein content and composition (Altenbach et al., 2002, 2003; Altenbach and Kothari, 2004;

Majoul et al., 2003, 2004; Triboui et al., 2003; Yang et al., 2002; Zahedi et al., 2004).

Previous controlled environment experiments compared the effects of post-anthesis N, in the form of NPK, on kernels produced under a moderate temperature regimen with 24 °C days and 17 °C nights (24/17 °C) with those produced under a regimen with high daytime temperatures but cool nights, 37 °C days and 17 °C nights (37/17 °C) designed to simulate high temperatures sometimes experienced during grain fill in the western United States (Altenbach et al., 2003). Effects of a more severe regimen of 37 °C days and 28 °C nights (37/28 °C) also were examined, since warm nights are often associated with periods of heat stress in the mid-western United States. Direct comparison of protein accumulation during grain development demonstrated that the addition of post-anthesis NPK increased the rate and duration of protein accumulation under both the 24/17 °C and the 37/17 °C regimens (Altenbach et al., 2003). Post-anthesis NPK had little effect on accumulation of starch, whereas the 37/17 °C and 37/28 °C regimens reduced accumulation of starch much more than protein. Direct comparisons of protein accumulation during grain development in kernels produced with and without post-anthesis NPK under the 37/28 °C regimen were not reported.

In this paper, the effect of post-anthesis NPK on protein accumulation, mixing and baking quality under the 37/28 °C regimen were evaluated. In addition, the effects of NPK on transcript levels for the gluten storage proteins and on profiles for accumulation of individual gliadin and glutenin storage proteins were examined under the 24/17 °C and 37/28 °C regimens to better understand the effects of temperature and mineral nutrition on regulation of storage protein accumulation and composition.

2. Materials and methods

2.1. Plant material and growth conditions

Plants of the US hard red spring wheat *Triticum aestivum* 'Butte86' were grown as described in Altenbach et al. (2003). Briefly, the plants were grown in a climate-controlled greenhouse with 16 h days and 8 h nights. Maximum daytime temperature was maintained at 24 °C for 5 h and minimum nighttime temperature at 17 °C for 11 h, with intermediate intervals at 21 °C (24/17 °C regimen). Plants were watered by drip irrigation with 0.6 g/l Plantex fertilizer (NPK, 20:20:20), receiving a total of approximately 0.1 g each of N, P and K per pot per day. Plants were grown in pots of 25 cm diameter \times 25 cm deep, at a density of seven plants per pot and pots were spaced closely to simulate a crop canopy. For the minus NPK treatment, pots were flushed with water at anthesis and then hand-watered until the grain was mature. All pots were weighed every 2 or 3 days and sufficient water was added as needed to maintain the soil at 80% of capacity. For the high temperature treatment, pots were transferred to a climate-controlled greenhouse maintained at 37 °C days and 28 °C nights, and fertilized by drip irrigation or hand-watered as above (37/28 °C regimen). Heads were collected at the same time of day at the indicated time points, grains excised,

and grains or extruded endosperm frozen in liquid N and stored at -80°C .

Four experiments were carried out between June, 2000 and July, 2002. For Experiment 1 heads underwent anthesis from June 16 to 20, 2000. Pots were divided into 2 groups of 13 each. One group was hand-watered after anthesis and one received NPK by drip irrigation until maturity. All plants were grown at $24/17^{\circ}\text{C}$. For Experiment 2 heads underwent anthesis from September 10 to 14, 2000. All pots were hand-watered from anthesis to maturity. Pots were divided into 2 groups of 18 each; one group was grown under the $24/17^{\circ}\text{C}$ regimen and one under the $37/28^{\circ}\text{C}$ regimen. For Experiment 3 heads underwent anthesis between June 1 and June 6, 2002. Pots were divided into 6 groups of 9 each. Three groups received post-anthesis NPK and three were hand-watered. All were grown under the $24/17^{\circ}\text{C}$ regimen. During development, 10 heads were collected from pots with post-anthesis NPK and 10 from pots without NPK, at 4–10 day intervals without regard to group. Only two heads were collected at day 40 and 44. Only fresh weight was determined, then endosperm was extracted for use in experiments not shown in this paper. At maturity, grain was collected and evaluated by group. For Experiment 4 heads underwent anthesis from June 25 to July 4, 2002. Pots were divided into 4 groups of 13 each. Two groups were grown under the $24/17^{\circ}\text{C}$ regimen and two under the $37/28^{\circ}\text{C}$ regimen; one group in each temperature regimen was hand-watered after anthesis and one received NPK by drip irrigation.

For all experiments, mature seeds from all pots in a treatment group were pooled. Average single kernel weight for mature grain was determined by sampling 100 grains from each pooled group. Samples of 100 g each were milled to flour and were analyzed for flour quality at the Hard Winter Wheat Quality Laboratory (US Department of Agriculture, Agricultural Research Service, Manhattan, KS).

2.2. Analysis of RNA transcript levels

RNA was prepared from grains from single heads collected at indicated times during grain fill. Transcripts were analyzed as in [Altenbach et al. \(2002\)](#). Probes specific for α -, γ -, and ω -gliadins, LMW-GS, and HMW-GS gene families and hybridization and detection methods were described in [Altenbach et al. \(2002\)](#).

2.3. Two-dimensional gel analysis of gluten proteins

The KCl-insoluble protein fraction was extracted from endosperm from single heads collected at the indicated times during grain fill. The methods for extraction and analysis by 2DE are described in detail in [Hurkman and Tanaka \(2004\)](#). Briefly, after extracting salt- and water-soluble proteins with 50 mM Tris-HCl, 100 mM KCl, 5 mM EDTA, pH 7.8, the remaining proteins, mainly glutenins and gliadins, were solubilized in 2% SDS, 10% glycerol, 50 mM DTT, 40 mM Tris-Cl, pH 6.8. For protein determination, triplicate samples were removed, precipitated with acetone, and protein quantified ([Lowry et al., 1951](#)). For gel analysis, acetone-precipitated proteins were resuspended

in 9 M urea, 4% NP-40, 1% DTT, and 2% ampholytes and 18 μg was loaded on the first dimension isoelectric focusing gel. After electrophoresis in the second dimension SDS gel, gels were stained with Coomassie G-250 (Sigma, St. Louis, MO), destained in water for 3 h and stored in ammonium sulfate to prevent fading of the ω -gliadin spots. Protein spots were excised and identified by mass spectrometry and N-terminal sequencing as described in [Vensel et al. \(2005\)](#). Computer software (Non-linear Dynamics Limited, Newcastle Upon Tyne, UK) was used to calculate normalized spot volumes (individual spot volume/total spot volume \times 100). Average and standard deviation for normalized volumes for individual spots in three replicate gels were determined for each developmental time point.

2.4. Measurement of fresh weight, dry weight, moisture, protein and starch

A minimum of three heads per time point were collected at the indicated intervals after anthesis, grains excised, and average single kernel fresh weight determined for each head. The grains were freeze-dried, average single kernel dry weight determined for each head, and average moisture determined by subtraction. Freeze-dried kernels from multiple heads were pooled and 5 g ground to a powder using a UDY mill (Udy Corporation, Fort Collins, CO). Protein content of the freeze-dried grain was determined in triplicate by Dumas nitrogen combustion analysis of 15 mg samples, using a Model FP-428 LECO nitrogen analyzer with a 10 ml gas collection tube (LECO Corporation, St. Joseph, MI), an EDTA standard, and a protein to N ratio of 5.7 (AACC Method 46-30, [American Association of Cereal Chemists, 2000](#)). Flour protein content was determined by NIR (AACC Method 39-11, [American Association of Cereal Chemists, 2000](#)) using the NIR Systems 6500 (NIR Systems, Silver Springs, MD). Standard error of prediction for moisture was 0.06% and for protein 0.13%. Starch content of 100 mg grain samples was determined in triplicate using the Megazyme total starch determination assay kit (Megazyme International, Bray, County Wicklow, Ireland; AACC Method 76-13, [American Association of Cereal Chemists, 2000](#)).

2.5. Micro-bread-making

Micro-bake tests using 10 g of flour were performed in duplicate at the Hard Winter Wheat Quality Laboratory (US Department of Agriculture, Agricultural Research Service, Manhattan, KS) according to previously described methods ([Shogren and Finney, 1984](#)) except that 50 mM ascorbic acid was used in place of potassium bromate and 0.10–0.13 g dry yeast was used per 10-g flour, adjusted to a 14% moisture basis. Optimum water absorption and dough mix time was used for each sample. Mixing tolerance was calculated as an angle measuring the downward slope in the mixing curve after peak mix time, and rated on a score of 0–6, where 0 is unsatisfactory, 2 is questionable, 4 is satisfactory, 5 is excellent and 6 is outstanding.

2.6. Statistical analysis

For Experiment 3, average single kernel weight and flour protein content was determined for grain from each pot, and treatment mean and variance determined by analysis of variance of the 6 groups and 2 treatments. For other experiments, standard deviations were determined as indicated.

3. Results

3.1. Effect of NPK on grain fill and flour protein content under two temperature regimens

Three sets of plants were grown with and without post-anthesis NPK under the 24/17 °C temperature regimen, in order to confirm prior observations that large increases in protein content were accompanied by small increases in single kernel weights. Application of post-anthesis NPK increased flour protein content from an average of $7.0\% \pm 0.4$ to $14.0\% \pm 0.9$. Single kernel fresh weight increased with NPK from an average of 47.8 ± 0.6 to 54.3 ± 1.3 mg kernel⁻¹ ($P < 0.001$). For this experiment, only single kernel fresh weight was determined throughout grain fill (Fig. 1). Under both regimens, maximum fresh weights were attained at about 30 days-post-anthesis (DPA) and kernels were mature by about 44 DPA. However, similar to results of Altenbach et al. (2003) the maximum fresh weight attained was slightly higher for kernels grown with NPK.

Grain development also was compared in the absence of post-anthesis NPK under two temperature regimens (Fig. 2). Dry weight, starch, and protein accumulation were plotted as functions of chronological time (Fig. 2A, C and E) and thermal time (Fig. 2B, D and F). The patterns of dry weight and starch accumulation were similar, with starch accounting for approximately 60% of total grain dry weight. Starch accumulation began earlier under the 37/28 °C regimen, commencing at 6 DPA, compared to 10 DPA under the 24/17 °C regimen (Fig. 2C). Duration of starch accumulation was reduced from 25 to 15 days and final amount of starch per grain was reduced by 44.7%, from 21.9 ± 1.3 to 12.1 ± 0.4 mg kernel⁻¹ for mature grain. Similarly, dry weight was reduced by 43.7%,

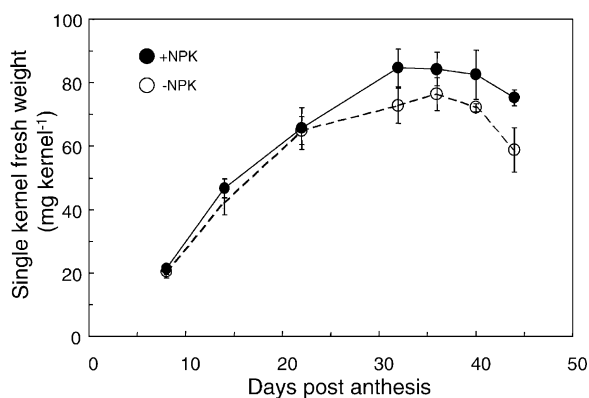


Fig. 1. Time course for accumulation of fresh weight under the 24/17 °C regimen in the presence (●) or absence (○) of post-anthesis NPK in Experiment 3. Standard deviation of data from 10 heads for each time point is indicated.

from 49.4 ± 2.6 to 27.1 ± 2.6 mg kernel⁻¹ for mature grain. The pattern of starch accumulation at 37/28 °C in the absence of post-anthesis NPK was nearly identical to that previously measured in the presence of post-anthesis NPK under the same temperature regimen (Altenbach et al., 2003). The duration of protein accumulation also was reduced, from 30 days under the 24/17 °C regimen to 15 days under the 37/28 °C regimen (Fig. 2E). However, the rate of protein accumulation was sufficiently increased under the 37/28 °C regimen so that final protein content of 4.6 ± 0.03 mg kernel⁻¹ was almost as high as that for grain produced under the 24/17 °C regimen, 5.1 ± 0.1 mg kernel⁻¹ for mature grain. Grain protein content was 10.5 ± 0.1 and $17.6 \pm 0.03\%$ for the 24/17 °C and 37/28 °C regimens, respectively. Notably, the rate and duration of protein accumulation for the 37/28 °C regimen without NPK was nearly identical to that published for the 37/28 °C regimen with NPK (Altenbach et al., 2003).

When the data were plotted in terms of accumulated thermal time, the rate of accumulation of starch and total dry matter were somewhat reduced by the 37/28 °C regimen, whereas the rate of protein accumulation was identical under the two temperature regimens. Duration of grain fill was identical under the 24/17 °C and 37/28 °C regimens, and accumulation of dry weight, starch, and protein all ceased at approximately 700 °C days under the two regimens. To verify the lack of effect of post-anthesis NPK on protein accumulation under the 37/28 °C regimen, the two temperature and NPK regimens were compared in a single experiment (Fig. 3). The maximum amount of dry weight appeared to be slightly higher in the presence than in the absence of post-anthesis NPK under the 24/17 °C regimen (Fig. 3A and B). The rate of accumulation of protein was much higher in the presence of post-anthesis NPK, so that final amount of protein per mature grain nearly doubled from 4.0 to 7.5 mg kernel⁻¹ (Fig. 3C and D).

As observed for Experiment 2 (Fig. 2), the duration in chronological time of dry weight accumulation in Experiment 4 (Fig. 3A) was much less under the 37/28 °C regimen. Accumulation of dry weight and protein began several days earlier under the 37/28 °C regimen. Maximum dry weight was attained by 15–20 DPA under the 37/28 °C versus 32–36 DPA under the 24/17 °C regimen, and maximum protein per kernel by 20–22 DPA versus 26–32 DPA. Much lower single kernel weights of 24.0 ± 1.8 and 22.8 ± 2.4 mg kernel⁻¹ were observed for the 37/28 °C regimen in the presence or absence of NPK, compared to 48.5 ± 2.4 and 44.9 ± 2.6 mg kernel⁻¹ for the 24/17 °C regimen with and without NPK.

When data were plotted as a function of accumulated thermal time, duration of accumulation of dry matter was shorter than for Experiment 2, ceasing or slowing greatly after 560 °C days under the 24/17 °C regimen, and after only 360 °C days under the 37/28 °C regimen. NPK had only a small effect on the pattern of dry matter accumulation under either temperature regimen.

The rate of protein accumulation was much higher in the presence than in the absence of NPK under the 24/17 °C regimen (Fig. 3C and D). In contrast, the rate and duration of protein accumulation and final protein content per grain were nearly identical in the presence or absence of post-anthesis NPK under

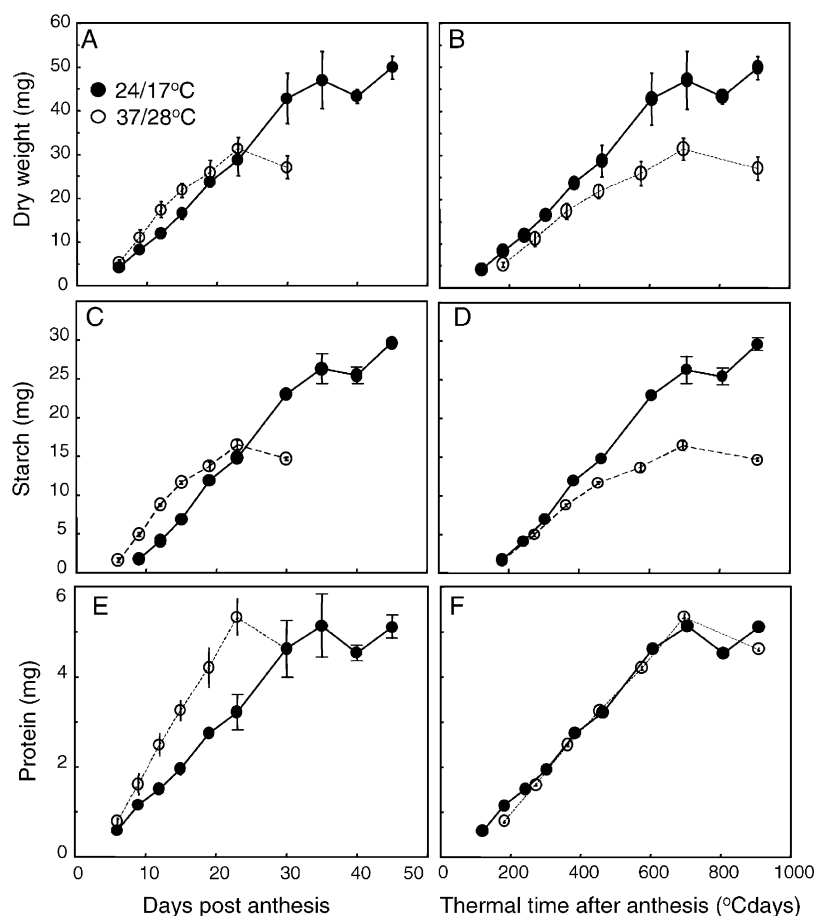


Fig. 2. Time course for accumulation of dry weight (A and B), starch (C and D) and protein (E and F) under the 24/17 °C (●) and 37/28 °C (○) regimens in the absence of post-anthesis NPK in Experiment 2. Data are plotted in chronological time as days-post-anthesis (A, C and E) and in thermal time above 0 °C (B, D and F). Standard deviation for dry weight is indicated for data from three heads for each time point. Standard deviation for starch and protein is for three replicates from a 5 g pool of dried grain for each time point.

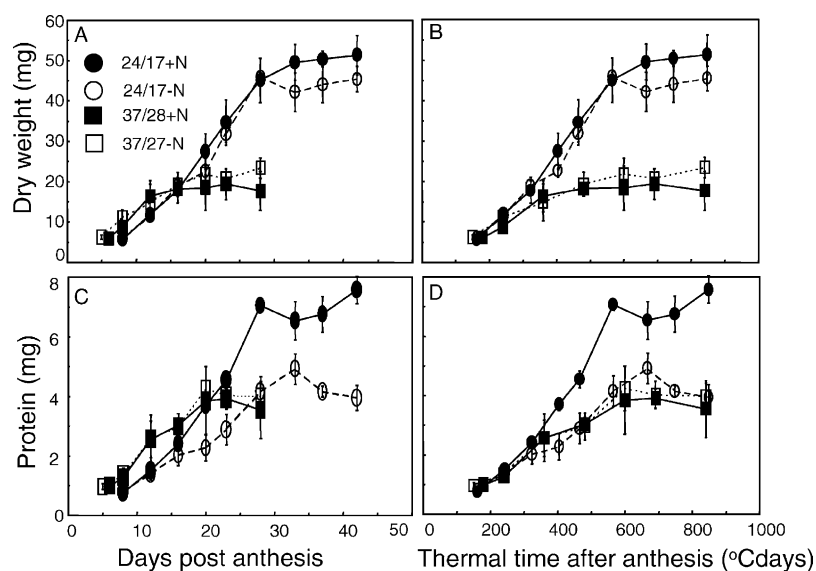


Fig. 3. Time course for accumulation of dry weight (A and B) and protein (C and D) under the 24/17 °C regimen (●, ○) and the 37/28 °C regimen (■, □), in the presence (●, ■, solid lines) or absence (○, □, dotted lines) of post-anthesis NPK in Experiment 4. Data are plotted in chronological time as days-post-anthesis (A and C) and thermal time above 0 °C (B and D). Standard deviations as in Fig. 2.

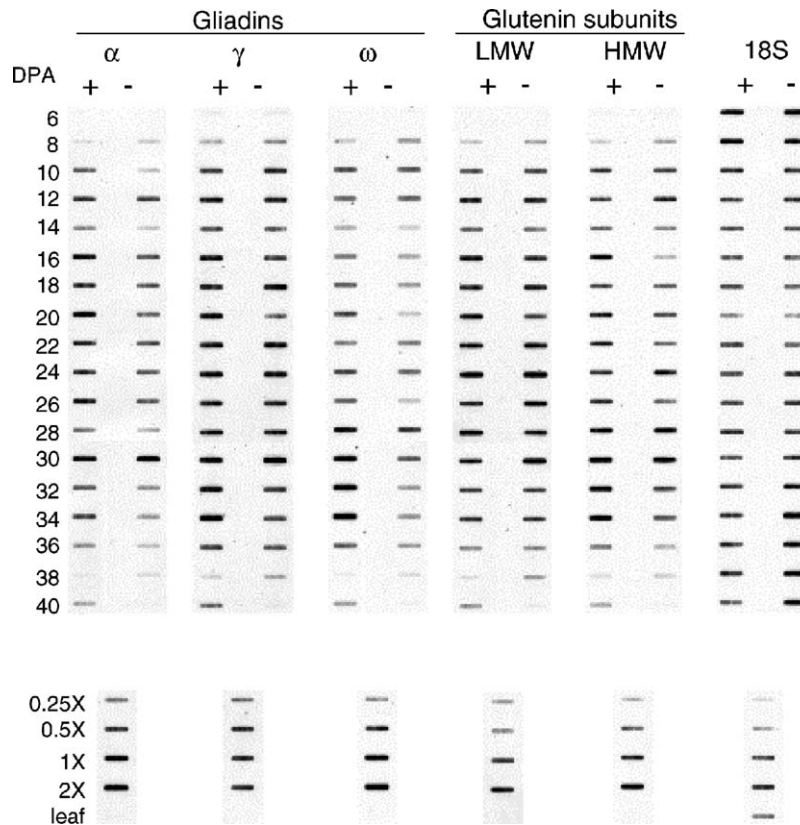


Fig. 4. Transcript accumulation profiles for gliadin and glutenin subunit genes expressed in developing wheat endosperm under a 24/17 °C regimen in the presence (+) or absence (–) of post-anthesis NPK in Experiment 1. Equal amounts of total RNA were hybridized to probes for the α -, γ - and ω -gliadin and LMW-GS and HMW-GS gene families or to a probe for 18S rRNA. At the bottom, hybridization of each probe to four dilutions of kernel RNA and 100 ng of leaf RNA are shown.

the 37/28 °C regimen (Fig. 3C and D). When plotted in thermal time, the pattern of accumulation of protein under the 37/28 °C regimens also was nearly identical to the pattern of accumulation of protein at 24/17 °C in the absence of post-anthesis NPK. Final amounts of protein per grain under the 24/17 °C regimen without NPK, 37/28 °C with NPK, and 37/28 °C without NPK regimens were very similar, 3.9, 3.6, and 4.0 mg kernel⁻¹, respectively (Fig. 3D), slightly more than half the 7.6 mg kernel⁻¹ accumulated under the 24/17 °C regimen with NPK. Although protein accumulation ceased by 560 °C days under the 24/17 °C regimen in the presence of NPK, it did not appear to cease until 600 °C days or more under the other three regimens.

3.2. Effects of NPK and high temperature on patterns of transcript levels for the gluten storage proteins

Since the gluten proteins encompass the bulk of the grain protein and are essential to bread making quality, accumulation profiles of transcripts for members of the HMW-GS, LMW-GS, and α -, γ - and ω -gliadin gene families were compared under those NPK regimens where rate or duration of protein accumulation differed in terms of chronological time. Transcript accumulation was compared under a 24/17 °C regimen with and without post-anthesis NPK (Experiment 1, Fig. 4). Details of fresh weight, dry weight, starch and protein accumulation for Experiment 1 are in Altenbach et al. (2003). Leaf

RNA and a dilution series with known amounts of kernel RNA were included in Fig. 4 to indicate the sensitivity of the assay. Total RNA was prepared from grains collected at 2 day intervals from 6 to 40 DPA. As previously reported (Altenbach et al., 2002), in the presence of post-anthesis NPK, transcript for all the major types of gluten proteins first began to accumulate at about 8 DPA and remained abundant until about 34 DPA, after which levels declined. The timing of transcript accumulation was similar with or without post-anthesis NPK. Transcript levels for LMW-GS and γ -gliadins were similar in the presence or absence of post-anthesis NPK throughout most of grain development. More variability was detected in transcript levels for the other classes of gluten protein, particularly the ω -gliadins, which were somewhat lower in the absence of post-anthesis NPK.

Transcript levels were also compared in kernels developing under the 24/17 °C and 37/28 °C regimens in the absence of NPK (Experiment 2, Fig. 5). The main effect of temperature was on the timing of gluten transcript accumulation, similar to that already reported in the presence of post-anthesis NPK. When expressed against chronological time, accumulation of transcripts for the gluten storage proteins began earlier under the 37/28 °C than under the 24/17 °C regimen and ceased earlier. Transcripts were first evident at 4–6 DPA under the 37/28 °C regimen and at 6–8 DPA under the 24/17 °C regimen. Transcripts disappeared after 22 DPA under the 37/28 °C regimen, but not until after 36

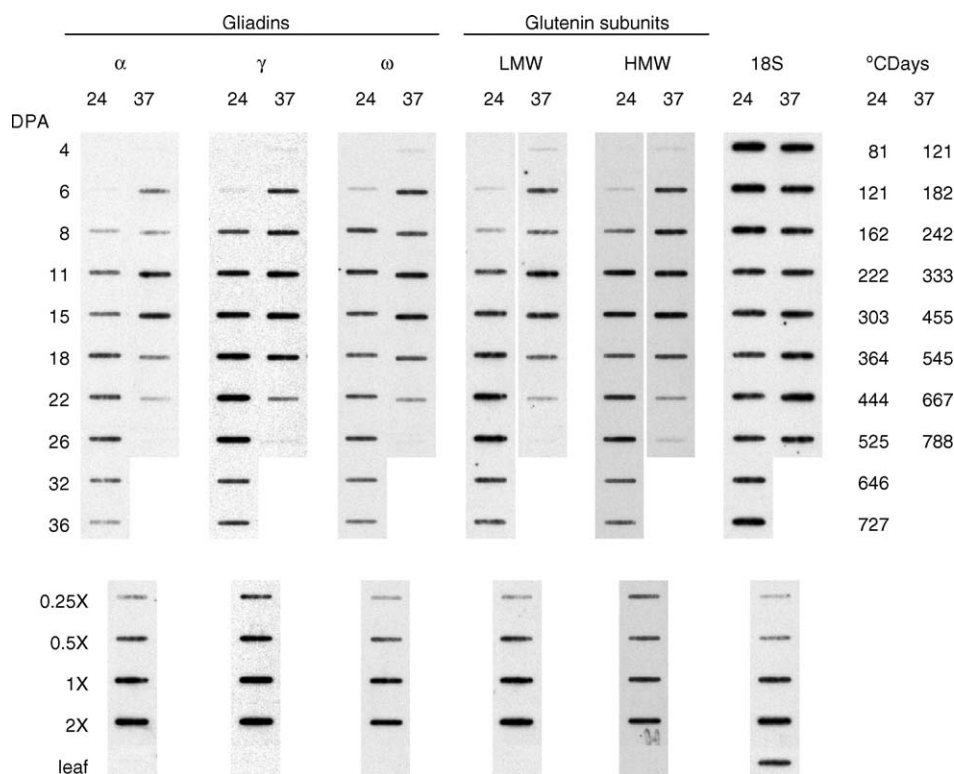


Fig. 5. Transcript accumulation profiles for gliadin and glutenin genes expressed in developing wheat endosperm under the 24/17 (24) or 37/28 °C (37) regimen in the absence of post-anthesis NPK in Experiment 2. Details are as in Fig. 4. Chronological time is indicated to the left as days post-anthesis (DPA); thermal time is indicated to the right for the two regimens as °C days.

DPA under the 24/17 °C regimen. For both temperature regimens, transcript began accumulating around 160–180 °C days and declined after 525–545 °C days, around the same time that protein accumulation ceased in Fig. 3D. The maximum level of transcript for each gluten protein type was similar under the two regimens.

3.3. Effects of NPK and high temperature on patterns of accumulation of the gluten proteins

The pattern of gluten storage protein accumulation under the four regimens was analyzed by 2DE at equivalent soft dough stages (Figs. 6 and 7). Despite the large differences in chronological rate and duration of protein accumulation under the different regimens, there was remarkable similarity in the overall protein pattern for grains produced under the four combinations of temperature and NPK when proteins from developmentally equivalent soft dough stages were compared, at 37 DPA for the 24/17 °C regimen (747 °C days, Fig. 6A and B) or 21 DPA for the 37/28 °C regimen (636 °C days, Fig. 6C and D). Protein spots that were analyzed in detail are indicated in Fig. 6A. One conspicuous difference between treatments was a decrease in the relative intensities of a number of protein spots under the 24/17 °C regimen in the absence of post-anthesis NPK, in particular, the three chromosome 1B encoded ω-gliadins ω-a, ω-b, and ω-c, and two chromosome 1D encoded ω-gliadins, ω-d, and ω-e (Fig. 6B). Relative intensities of a few other spots also decreased, including those for proteins that have not been iden-

tified with certainty by mass spectrometry or N-terminal amino acid sequencing.

To illustrate specific effects of temperature or NPK on patterns of protein accumulation, gluten protein extracts were prepared from endosperm from heads collected between 8 and 37 DPA under the 24/17 regimen or 6–21 DPA under the 37/28 °C regimen. The insoluble gluten fraction was analyzed by 2DE and relative spot densities were determined at each time point. Data for the earlier stages of grain fill (8 DPA for the 24/17 °C and 6 DPA for the 37/28 °C regimens) are not included because the major spots were not gliadins or glutenins. For α-, γ- and ω-gliadins and LMW-GS, two spots that could be clearly identified throughout the indicated time period were chosen for the analysis. Criteria were that there was no overlap with a different protein early in grain fill and at least one trypsin fragment gave a satisfactory match to known database sequences. All five HMW-GS were analyzed. The spot volume data are plotted against thermal time (Fig. 7).

Under the 24/17 °C regimen, spots α-b (Fig. 7B), ω-a (Fig. 7E) and ω-b (Fig. 7F), were at near maximum levels by 263 °C days (13 DPA). Normalized volumes of ω-a and ω-b decreased in the absence of NPK, and by 748 °C days (37 DPA) their relative spot volumes were nearly two-fold less in the absence of post-anthesis NPK. Similar results were found for ω-c (not shown). Normalized volumes for the other spots analyzed in Fig. 7 increased gradually under the 24/17 °C regimens. The α-a spot reached near maximum by 363 °C days (18 DPA, Fig. 7A), the γ-a spot by 464–565 °C days (23–28 DPA,

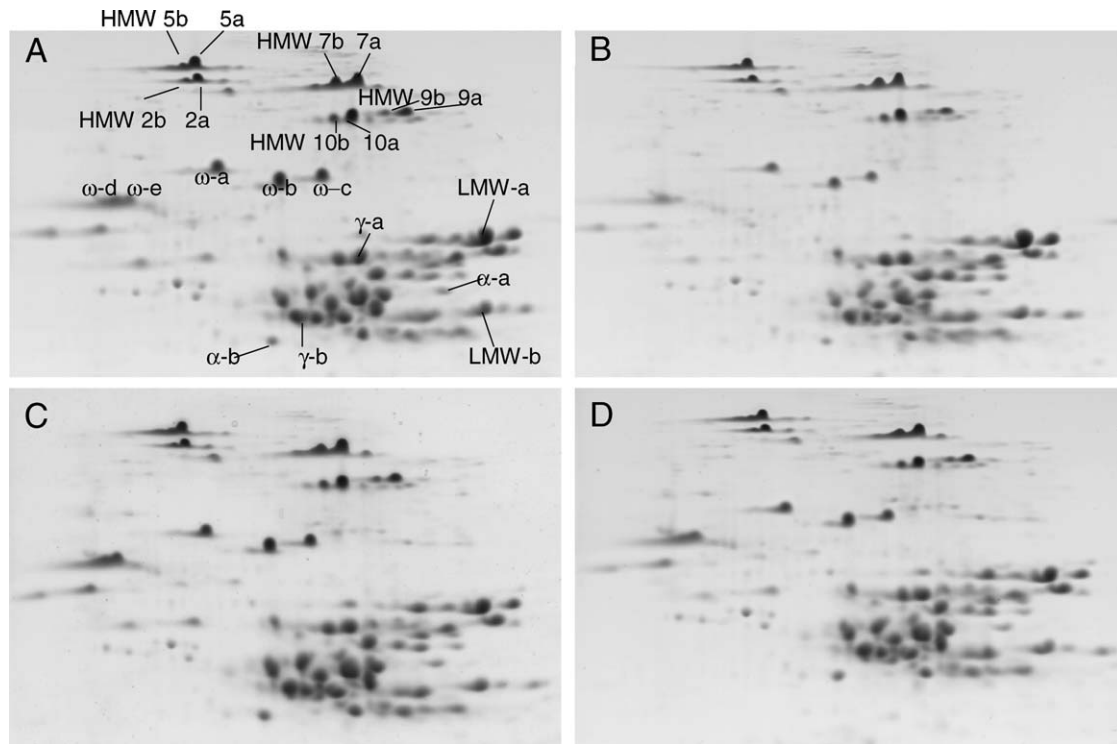


Fig. 6. Two-dimensional gels (2DE) of insoluble protein fractions from endosperm collected from 37 DPA grains produced under the 24/17 °C regimen (A and B) or from 21 DPA grains produced under the 37/28 °C regimen (C and D) in the presence (A and C) or absence (B and D) of post-anthesis NPK in Experiment 4. The two major spots in the spot-trains for the HMW-GS encoded on chromosomes 1A, 1B, and 1D are indicated as HMW 2a and 2b (HMW-GS Ax2*), HMW 5a and 5b (HMW-GS Dx5), HMW 7a and 7b (HMW-GS Bx7), HMW 9a and 9b (HMW-GS By9) and HMW 10a and 10b (HMW-GS Dy10). The 3 distinct spots for ω -gliadins encoded on chromosome 1B are labeled ω -a, ω -b, and ω -c, and 2 distinct spots for ω -gliadins encoded on chromosome 1D as ω -d and ω -e. Two LMW-GS spots are indicated (LMW-a and LMW-b), two α -gliadins (α -a and α -b) and two γ -gliadins (γ -a and γ -b). Proteins were identified by mass spectrometry based on their similarity to the following sequences in the Swiss Prot database and the indicated e-values: α -a, Q9M4L8, 3.0×10^{-5} ; α -b, Q41529, 6.8×10^{-3} ; γ -a, Q94G91, 1.2×10^{-3} ; γ -b, Q9XE0, 4.7×10^{-3} ; LMW-a, Q8GU18, 3.8×10^{-21} ; LMW-b, P94021, 1.6×10^{-3} ; HMW-2a, Q41553, 3.30×10^{-31} and HMW-2b, Q41553, 8.7×10^{-3} ; HMW-5a, P10388, 2.0×10^{-3} and HMW-5b, P10388, 8.9×10^{-7} ; HMW-7a, Q42451, 1.2×10^{-3} and HMW-7b, Q42451, 3.5×10^{-3} ; HMW-9a, Q03871, 1.3×10^{-3} and HMW-9b, Q03871, 3.8×10^{-3} ; HMW-10a, P10387, 6.1×10^{-29} and HMW-10b, Q93WM1, 6.7×10^{-5} . The ω -gliadins were identified by N-terminal and internal sequence (Dupont et al., 2000).

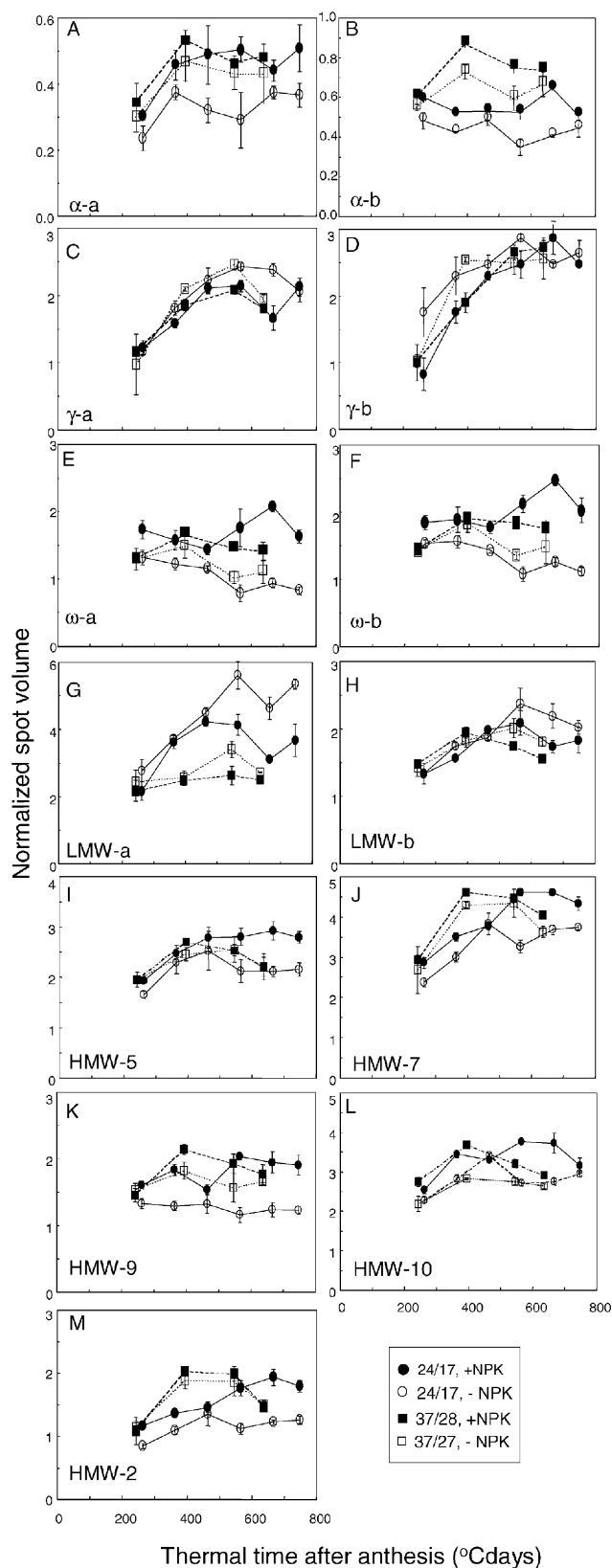
Fig. 7C) and the γ -b spot by 464–667 °C days (23–33 DPA, Fig. 7D).

The LMW-a spot reached maximum proportion of total protein by 464 °C days (23 DPA) in the presence of NPK but continued to increase in proportion until 565 °C days (28 DPA) in the absence of NPK (Fig. 7G). In contrast to the other protein spots analyzed for this paper, under the 24/17 °C temperature regimen the LMW-a spot density was somewhat higher in the absence than in the presence of post-anthesis NPK by 747 °C days (37 DPA, Fig. 7F). LMW-b followed a similar pattern but the difference in response to NPK was smaller (Fig. 7H).

The HMW-GS resolved into spot trains, so values for the two largest spots in the spot train were summed for each HMW-GS. HMW-GS Dx5 (HMW-5, Fig. 7I) and HMW-GS By9 (HMW-9, Fig. 7K) reached maxima by 363 °C days (18 DPA). HMW-Dy10 (HMW-10, Fig. 7L) reached a maximum by 464 °C days (23 DPA). HMW-GS Bx7 (HMW-7, Fig. 7J) gradually increased until 565 °C days (28 DPA) in the presence of NPK, as did HMW-GS Ax2* (HMW-2, Fig. 7M). Normalized volumes of all the HMW-GS were somewhat lower under the 24/17 °C minus NPK regimen; the biggest decreases were for HMW-2, HMW-5 and HMW-9.

In terms of chronological time, all of these proteins began to accumulate earlier under the 37/28 °C than under the 24/17 °C regimen. The relative spot volume patterns were generally similar, however, when treated in accumulated thermal time. Relative spot volumes for the α -a spot at 37/28 °C, with or without NPK, followed a pattern nearly identical to that for the α -a spot at 24/17 °C in the presence of NPK, whereas the α -b spot volumes were somewhat higher at 37/28 °C than at 24/17 °C. The spot volume patterns for the γ -a and γ -b spots at 37/28 °C were nearly identical to those at 24/17 °C under the same NPK regimen, as were the ω -a and ω -b spot volume patterns. Under the 37/28 °C regimen the normalized volumes for LMW-a were lower than those observed at 24/17 °C, whereas those for LMW-b were similar under both temperature regimens. The HMW-GS spot volumes were similar to those observed at 24/17 °C, except that HMW-2, 7 and 10 appeared to peak earlier at 37/28 °C.

Changes in normalized volume throughout development were small for most of the spots analyzed. For nearly mature grain at 747 °C days (37 DPA) under the 24/17 °C regimen, the largest changes in response to NPK were decreases from 1.6 to 0.8 for ω -a, 2.0 to 1.1 for ω -b, 1.7 to 1.2 for HMW-2, 1.9 to 1.2 for HMW-9, and 2.8 to 2.2 for HMW-5 and the increase from 3.7 to



5.4 for LMW-a. At the last time points examined, 747 °C days (37 DPA) under the 24/17 °C regimen with NPK, compared to 636 °C days (21 DPA) under the 37/28 °C regimen with NPK, the largest changes in response to temperature were a decrease from 3.7 to 2.5 for LMW-a and an increase from 0.5 to 0.8 for the α-b spot.

3.4. Effect of the temperature and NPK regimens on some milling and baking parameters

Grain from Experiments 2–4 was milled and 10 g loaves were mixed and baked in order to assess the effects of the treatments on milling and baking quality (Table 1). One measurement was made for each flour sample, and the following trends were observed for the total 12 samples. NPK had little effect on flour yield or bushel weight. The 37/28 °C regimen decreased grain size and thus bushel weight and milling yield. Mixing tolerance tended to be highest for high protein flours from grain produced under the cool temperature regimen with post-anthesis NPK, with scores of 3–4, and lower for the flours produced without NPK under either temperature regimen, mainly with scores of 2. The lowest score was 1, for flour from grain produced under the 37/28 °C regimen with NPK. Despite the low mixing tolerance scores of 1–2 for high-protein flour from plants grown under the high temperature regimen, loaf volume was proportional to flour protein content and not decreased by the high temperature regimen (Fig. 8). When protein content increased because of post-anthesis NPK or heat, loaf volume also increased. The primary purpose of the experiments in this paper was to collect material for developmental studies. Sufficient grain was produced to collect limited milling and baking data for the individual experiments, but there was not enough material for statistical replicates except for Experiment 3. However, the data for the 12 flour samples summarized in Table 1 and Fig. 8 illustrate the effects of the treatments on flour quality. Similar effects of the 37/28 °C regimen on mixing tolerance were found in other experiments (data not shown).

4. Discussion

Temperature and mineral nutrition are major environmental factors that are reported to influence flour bread making quality through effects on protein amount and composition. In this study, we characterized the effect of post-anthesis temperature and NPK regimens on protein accumulation, transcript levels for gluten protein types, proportions of individual gluten proteins resolved by 2DE, and milling and baking parameters for

Time course for accumulation of individual gliadins and glutenins under the 24/17 °C regimen (●, ○; solid line) and the 37/28 °C regimen (■, □; dotted line), in the presence (●, ■) or absence (○, □) of post-anthesis NPK in Experiment 4. Data are plotted as thermal time above 0 °C, starting at anthesis. Protein was extracted from single heads harvested at the indicated times. Normalized spot volumes were calculated for (A) α-a (B) α-b; (C) γ-a; (D) γ-b; (E) ω-a (F) ω-b; (G) LMW-a; (H) LMW-b; (I) HMW 5-a plus 5-b; (J) HMW 7-a plus 7-b (K) HMW 9a plus 9b; (L) HMW 10a plus 10b and (M) HMW 2-a plus 2-b. Data shown are average and standard deviation for three replicate gels for each time point.

Table 1

Milling, mixing and baking parameters for flour from plants grown in Experiments 2–4 under different temperature and NPK regimens

Experiment	Temperature (°C)	NPK	Bushel weight (kg)	Flour yield (%)	Moisture (%)	Protein (%)	Mix time (min)	Mix tolerance	Loaf volume (ml)
2	24/17	–	64.0	67.9	14.2	8.8	2.25	2	64
2	37/28	–	n.d.	61.1	14.0	14.9	2.25	2	92
3	24/17	–	61.2	68.9	13.7	7.3	2.50	3	52
3	24/17	–	61.2	66.8	13.8	7.1	3.50	2	53
3	24/17	–	60.7	66.8	14.0	6.5	2.38	2	50
3	24/17	+	63.0	68.2	14.0	14.4	2.13	4	84
3	24/17	+	62.6	69.1	14.2	14.6	2.00	3	84
3	24/17	+	63.4	69.2	14.3	13.0	2.50	4	78
4	24/17	–	61.3	68.7	14.1	7.7	3.38	2	53
4	24/17	+	62.5	68.2	14.3	14.0	2.00	3	84
4	37/28	–	51.7	61.7	13.6	15.9	3.38	2	92
4	37/28	+	51.2	59.5	13.6	17.7	3.00	1	90

One determination of mixing time, mixing tolerance and 10 g loaf volume was made for each sample. n.d., not determined.

a single variety of hard red spring wheat. The eventual goal is to understand the molecular basis for effects of environment on flour quality.

The rate and duration of protein accumulation changed in response to temperature or NPK levels. Rates were increased by addition of NPK under the 24/17 °C regimen, whether data were plotted in terms of chronological or thermal time. When data were plotted in terms of thermal time, rate and duration of protein accumulation were the same for plants grown under the 24/17 °C regimen without NPK and under the 37/28 °C regimen with or without NPK. Duration in chronological time was decreased by the 37/28 °C regimen. Protein amount per kernel doubled under the 24/17 °C regimen when wheat plants were supplied with post-anthesis NPK (Altenbach et al., 2003, and this paper), but was unchanged by the 37/28 °C regimen with or without NPK. Similar results were reported by Zahedi et al. (2004), who found that addition of post-anthesis N under a 30/25 °C regimen did not increase the rate or duration of protein accumulation. The data suggest that kernels were able to make use of the

additional post-anthesis NPK to increase protein levels under the 24/17 °C regimen but not under the 37/28 °C regimen. In support of this, we observed that heads matured before leaves senesced on plants grown under the 24/17 °C regimen with post-anthesis NPK, suggesting there was additional uptake and incorporation of post-anthesis nitrogen into the leaves, as reported by Spiertz and Ellen (1978). In contrast, leaves senesced before heads matured in the absence of post-anthesis NPK. Under the 37/28 °C regimen, leaf senescence preceded maturation of heads under both NPK regimens (Altenbach et al., 2003). It is also possible that shifting the temperature to the 37/28 °C regimen enhanced remobilization of nitrogen from leaves and stems.

Despite large effects of NPK or temperature on rate and chronological duration of protein accumulation, transcript levels for the major gluten protein types were very similar throughout development under the different regimens. This is in agreement with the similarity in duration of protein accumulation in thermal time. Minor changes in the levels of gluten protein transcripts were observed in response to NPK. The most consistent were small decreases in transcript for ω -gliadins in the absence of NPK under the 24/17 °C regimen. Differences in the chronological timing of transcript accumulation, but not maximum transcripts levels, were observed when grains from the two temperature regimens were compared in the absence of NPK. Analysis of gluten proteins by 2DE suggests that changes in gluten protein composition also were relatively small. In fact, the overall similarity in gliadin and glutenin subunit composition of grains produced over 45 days at 24/17 °C with or without NPK to those produced in only 26 days at 37/28 °C is quite remarkable and suggests that the synthesis of the gluten proteins is tightly controlled. When the relative proportions of selected gluten proteins were compared throughout grain development, the ω -gliadins exhibited the greatest response to NPK, a two-fold decrease in normalized spot volume under the minus NPK regimen at 24/17 °C. However, some α -gliadins and HMW-GS also showed reduced spot volumes in late stages of development in the absence of NPK, whereas the LMW-a spot increased by 20% in the absence of NPK. Several other groups have reported changes in gluten protein composition in mature grain in response to fertilizer, nitrogen or sulfur levels, with the largest

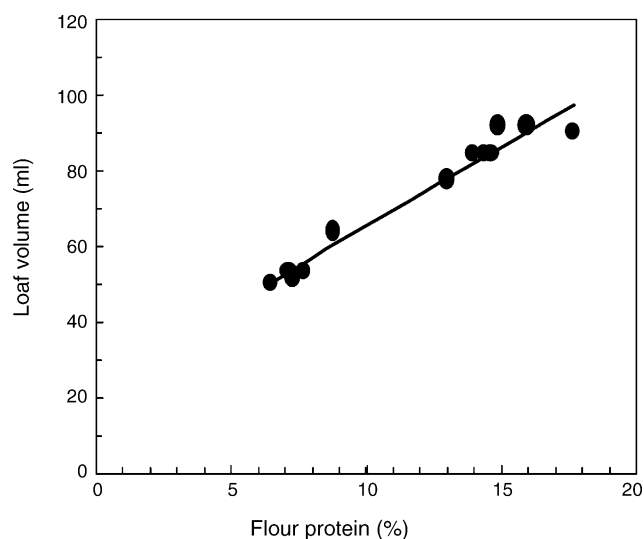


Fig. 8. Loaf volume as a function of flour protein percentage for the experiments shown in Table 1. Line fit by linear regression analysis, $r^2 = 0.96$; $y = 4.18x + 23.53$; one- and two-tailed $P < 0.001$.

changes being reported for the ω -gliadins (Wieser and Seilmeier, 1998; Wrigley et al., 1984).

The protein composition of wheat gluten is highly complex. Six highly repetitive and closely related genes encode HMW-GS, 20 or more each encode LMW-GS, α -gliadins and γ -gliadins, and several more encode ω -gliadins (Anderson et al., 1997; D'Ovidio and Masci, 2004; DuPont et al., 2000; Payne, 1987). Because of this complexity, accurately measuring changes in gluten types or in individual proteins is not trivial. Other studies report only total changes in gliadin types and LMW-GS, although several have analyzed individual HMW-GS (Wieser and Zimmermann, 2000; Carceller and Aussenac, 1999). This report illustrates the power of 2DE to detect changes in individual proteins within each gliadin and LMW-GS family. It should be noted that relatively small changes in protein amount, for example an increase from 12 to 13% in flour protein content, can be significant to the end-user. Changes in composition, such as in the ratio of gliadins to glutenins is also thought to alter the viscoelastic properties of flour doughs. Because of complex interactions between the different gluten proteins, it also is possible that a 20% change in the amounts of key glutenin subunits will impact bread-making quality. Such subtle changes in transcript or protein levels are difficult to measure convincingly, but warrant further investigation.

A preliminary assessment indicates that loaf volume was correlated with protein amount, whereas mixing tolerance was low both for flour from the low-protein grain produced at 24/17 °C and the high-protein grain produced at 37/28 °C. Additional experiments illustrate the deleterious effects of the 37/28 °C regimen on mixing tolerance (DuPont and Chung, unpublished data) but are not shown in this paper. Mixing time was variable, in part because it may be difficult to assess this parameter for flour samples with protein contents lower than 8%, as was produced under the 24/17 °C regimen without NPK. Also, mixing time for Butte86 tends to be low. Our results are in agreement with other reports of negative effects of high temperature on mixograph or extensigraph measurements (Blumenthal et al., 1995; Corbellini et al., 1997; Gibson et al., 1998; Randall and Moss, 1990; Stone et al., 1997). Butte86 seems to be somewhat tolerant to high temperatures, however, since it was necessary to treat the plants to the 37/28 °C regimen from anthesis to maturity to obtain a large decrease in mixing tolerance. The results also are in agreement with findings that application of N fertilizer late in the growing season can make a substantial contribution to grain protein content, with beneficial effects on bread making quality (Lotfollahi et al., 1997; Randall and Moss, 1990; Wuest and Cassman, 1992a,b).

5. Conclusions

Under moderate temperatures, the addition of post-anthesis NPK increased protein content, mainly by affecting the rate of protein accumulation. The increase in protein was accompanied by changes in relative spot volume for individual gluten storage proteins, with increases in the S-poor ω -gliadins and HMW-GS, as well as in the S-rich α -gliadins, little effect in the S-rich γ -gliadins, but decreases in the S-rich LMW-GS.

Similarly, transcript for the ω -gliadins, α -gliadins and HMW-GS declined somewhat after removal of fertilizer at anthesis, whereas transcript for LMW-GS and γ -gliadins was maintained at a similar level under both fertilizer regimens. In contrast, under a high temperature regimen with hot days and warm nights, post-anthesis NPK had little effect on protein accumulation in the developing grain, altering neither the rate nor the duration of protein accumulation. Similarly, few changes in protein composition of the nearly mature grain were detected in response to the high temperature regimen, although a decrease in relative spot volume for a major LMW-GS was noted. The application of post-anthesis NPK increased protein content and improved quality parameters of the resulting flour, when applied under the moderate temperature regimen. Flour protein content was high under the high temperature regimen, whether or not NPK was added. Loaf volume was high for all of the high protein flours, but mixing tolerance was good only for the high protein flour produced under the 24/17 °C regimen. Further research is required to discover whether effects of temperature on individual gluten proteins, particularly the major LMW-GS spot, are related to decreased mixing tolerance.

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Mention of a specific product name by the United States Department of Agriculture does not constitute an endorsement and does not imply a recommendation over other suitable products.

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